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Analysis of Two Gene Clusters Involved in the Degradation of 4-Fluorophenol by *Arthrobacter* sp. Strain IF1

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*Arthrobacter* sp. strain IF1 is able to grow on 4-fluorophenol (4-FP) as a sole source of carbon and energy. To clone the 4-FP degradation genes, DNA libraries were constructed and screened with a probe obtained by PCR using primers designed on the basis of conserved regions of aromatic two-component monooxygenases. Sequencing of positive clones yielded two gene clusters, each harboring a gene encoding a monooxygenase with high sequence similarity to the oxygenase component of 4-nitrophenol and 4-chlorophenol monooxygenase systems. Both these monooxygenase genes were differentially expressed during growth on 4-FP, as revealed by Northern blotting and reverse transcription-PCR. One cluster also contained a gene for a flavin reductase. The monooxygenase and reductase were purified from *Escherichia coli* cells expressing the corresponding genes, and together they catalyzed NADH-dependent hydroxylation and dehalogenation of 4-halophenols. The results indicate that strain IF1 transforms 4-FP to hydroquinone by a two-component monooxygenase system of which one component provides reduced flavin adenine dinucleotide at the expense of NADH and the other catalyzes para-hydroxylation of 4-FP and other 4-substituted phenols.

Halogenated phenols are used as building blocks in the synthesis of pharmaceuticals, agrochemicals, and performance materials. They also occur frequently as pollutants in water and soil and may cause serious environmental problems. Some soil microorganisms have evolved biodegradation pathways that allow growth on these compounds (9, 18, 26, 47). Most studies of such pathways have been conducted with organisms that degrade and dehalogenate chlorinated phenols. Since the 1990s, the industrial use of fluorinated compounds has been growing (20, 45).

Pathways for the biodegradation of fluorinated compounds and the enzymes catalyzing defluorination have scarcely been examined, although some routes are known (34). The cleavage of the carbon-fluorine bond is especially interesting in view of its kinetic stability and high bond energy. Defluorination of fluoraromatics can occur prior to ring cleavage, e.g., via an oxygenase that defluorinates fluorobenzoate (11, 36) or fluorobenzene (8). In other cases, defluorination occurs after ring cleavage via the formation of fluorinated muconolactones (42), which can be produced from 4-fluorobenzoate (19) and fluorobenzene (8) via 4-fluorocatechol. Bacterial and fungal phenol hydroxylases can convert fluorophenols to fluorocatechols or fluoropyrogallols, which are metabolized to fluoromuconic acids by ring cleavage dioxygenases (5, 6). Defluorination of 4-fluorophenol (4-FP) prior to ring cleavage in a strain of *Arthrobacter* has recently been described by us, but the enzymatic basis of defluorination was not solved (13).

For chlorinated phenols, two main metabolic routes have been described. Pathways in which the chlorophenol is oxidized to a substituted catechol, in some cases with partial dehalogenation, followed by ortho-cleavage of the aromatic ring and post-ring cleavage dehalogenation, occur in bacteria that degrade monochlorophenols (18, 21, 47, 48). On the other hand, routes in which the substituted phenol is converted via hydroquinone (or a substituted hydroquinone) to maleylacetate are also known, mainly in organisms that grow on polyhalophenols (28, 30, 31, 38, 44, 50). The further aerobic metabolism of hydroquinone may proceed via direct ring fission (7, 32) or via hydroxylation to hydroxyhydroquinone (1,2,4-trihydroxybenzene) (12), which undergo ring fission by an intradiol dioxygenase (25, 29, 33, 35). Genes for the latter hydroquinone degradation route have been cloned from *Cupriavidus necator* (formerly *Ralstonia eutropha*) strain JMP134 and *Ralstonia pickettii* DTP0602, both of which grow on 2,4,6-trichlorophenol (20, 30, 31), and from a strain of *Sphingobium chlorophenolicum* that can grow on pentachlorophenol (9, 37).

In the present paper, we report the characterization of two 4-FP catabolic gene clusters from *Arthrobacter* sp. strain IF1 (13) and characterize the expression and function of the two-component flavin monooxygenase genes that are involved in the initial steps of 4-FP degradation. The organism was isolated on the basis of its ability to utilize 4-FP as a carbon source for growth (13).

**MATERIALS AND METHODS**

*Bacterial strains and culture conditions.* *Arthrobacter* sp. strain IF1 (13) was grown in Luria-Bertani (LB) medium or in a synthetic medium (13) at 30°C. *Escherichia coli* BL21(DE3) (Stratagene) was grown in LB medium, and when necessary, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μg/ml of ampicillin were added.

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‡ Supplemental material for this article may be found at http://aem.asm.org.

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Isolation and analysis of DNA. Small plasmid DNA was isolated as described by Sambrook et al. (41). Genomic DNA was isolated from strain IF1 as previously described (14), and plasmid DNA was isolated with a large-contract isolation kit from Qiagen.

The following degenerate primer set was used for the initial amplification and cloning in pGEM-T Easy (Promega) of segments of the 4-FP monooxygenase genes: primer 1 (5'-AAGCTGCAAGACGGCTG-3'; for sequence NVATHP) and primer 2 (5'-GGCCGGATTCTTAAACCRT-3'; reverse complement for sequence (ND)VFPWS). PCR was performed with these primers in 50 μl reaction mixtures containing 4 μl of a deoxycholine triphosphate mixture, 0.5 μl ExTaq DNA polymerase (Takara Bio, Shiga, Japan), and 50 ng of strain IF1 genomic DNA. PCR schedules were as follows: 94°C for 8 min; 34 cycles consisting of 94°C for 30 s, a gradient from 45°C to 55°C for 40 s, and 72°C for 50 s; and a final incubation at 72°C for 3 min.

Cloning and sequencing of monooxygenase genes. Genomic DNA was separately digested by ApaI and BamHl subjected to gel electrophoresis, and transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Hertules, CA) by blotting. Putative monooxygenase sequences were detected with a digoxigenin hybridization system (Roche Diagnostics, Mannheim, Germany) as described previously (24), using a probe obtained by labeling of PCR products that were produced by amplification of genomic DNA with the degenerate primers mentioned above. A 5-kb band was detected with genomic DNA that was treated with AplAI, and fragments of this size were cloned into pBluescript II KS(+) (Stratagene) to produce library A. Fragments of 9 kb were detected with BamHl-restricted DNA and were cloned into pHSg397 (Takara) to produce library B. For screening, the libraries were transformed into E. coli cells, and transformants were inoculated into several Falcon tubes containing 2 ml of LB medium and chloramphenicol (for pHSg397) or ampicillin [for pBluescript II KS(+)]. After overnight growth at 30°C, DNA was isolated and screened for the presence of the 4-EP monooxygenase gene by PCR with the primers used earlier for the preparation of the probe. Positive cultures were plated, and colonies were screened again by PCR. DNA was isolated from the positive clones, cloned into pUC19 (Takara), and used for sequencing.

Dideoxy sequencing was done using an ABI Prism BigDye ready reaction kit and an ABI sequencer, model 3700. Sequences were analyzed as described previously (24).

Analysis and mRNA. Cellular mRNA levels were determined by Northern blotting and reverse transcription-PCR (RT-PCR). Cells were grown in LB medium, collected at an optical density at 600 nm (OD600) of 0.7, washed with saline-phosphate buffer (pH 7.0), and resuspended in a mineral medium (MM) (13) to an OD600 of 3.5. The cells were distributed to test tubes and incubated with a carbon source (10 mM glucose, 10% glycerol, 10 mM succinate, 1 M 4-FFP, or 1 M 4-nitrophenol) for 6 h at 30°C with shaking. Then cells were collected and disrupted (23), and total RNA was extracted using an Isogen RNA extraction kit (Nippon Gene, Toyama, Japan).

For Northern hybridization, RNA was transferred to a membrane by capillary blotting, followed by hybridization with labeled probes prepared from PCR products of the fpdA1 and fpdA2 open reading frames (ORFs) with the primer pairs that were also used for making the expression constructs. The primer sequences were as follows: fpdA1/A2-5′/3′, which amplified a 1.189-bp region from fpdA1, and primer set Bam forward, 5′-CGTCGGGAGAGGTACGCTGA-3′; reverse, 5′-TGGCGGCTAGCTGCGGCGGCT-3′, which amplified a 1.898-bp region from fpdA2, and primer set Bam forward, 5′-GGCTGAGAGGTACGCTGA-3′; reverse, 5′-TGGCGGCTAGCTGCGGCGGCT-3′, which amplified a 1.999-bp region from fpdA2. Amplification was performed with 100-fold-diluted cDNA samples and ExTaq DNA polymerase (Takara Bio, Shiga, Japan) with the following schedule: 94°C for 2 min; 25 cycles consisting of 94°C for 20 s, 67°C for 20 s, and 72°C for 50 s; and a final incubation at 72°C for 5 min. DNase-treated RNA samples that were not subjected to reverse transcriptase treatment were used as controls in PCRs to verify the absence of contaminating genomic DNA. PCR products were analyzed on a 1.0% agarose gel.

Expression of fpd genes in E. coli. The nucleotide sequences of fpdA1, fpdA2, and fpdB were amplified with PCR primers (sequences mentioned above) and cloned into pET17b (Novagen) as translational fusions in the NdeI restriction site of the vector. E. coli BL21(DE3) was used for expression.

Purification of 4-EP monooxygenase (FpdA2). The 4-EP monooxygenase (FpdA2) was purified from E. coli BL21(DE3)pETfpdA2. Cells were grown in LB medium containing ampicillin until the OD600 reached 0.5. IPTG was then added (0.5 mM), and the culture was incubated overnight at 20 to 22°C with shaking. Cells were harvested by centrifugation, washed twice with TEMG buffer (50 mM Tris⋅HCl, pH 7.5, 0.5 mM EDTA, 1 mM β-mercaptoethanol, 5% glycerol), resuspended in the same buffer, and disrupted by sonication. After centrifugation (at 40,000 × g for 60 min), the extract was loaded onto a DEAE Sepharose column (bed volume, 60 ml) pre-equilibrated with TEMG buffer. FpdA2 was eluted with a linear gradient of 0 to 0.5 M (NH4)2SO4 in TEMG buffer, concentrated by ultrafiltration (Amicon YM-30 membrane), and separated on a hydroxyapatite column (50 ml) using 10 to 400 mM potassium phosphate (pH 7.0) containing 1 mM β-mercaptoethanol and 5% glycerol. FpdA2 was concentrated by ultrafiltration and stored at −20°C.

Purification of flavin reductase (FpdB). Flavin reductase was purified from E. coli BL21(DE3)pETfpdB, cultivated, induced, and lysed as described above for FpdA2. The cell extract was fractionated on a DEAE Sepharose column, after which FpdB protein was concentrated and dialyzed against 1.5 M (NH4)2SO4 in TEMG buffer, concentrated by ultrafiltration (Amicon YM-30 membrane), and separated on a hydroxyapatite column (50 ml) using 10 to 400 mM potassium phosphate (pH 7.0). Enzyme activity corresponds to 1 μmol of 4-EP converted per min.

Enzyme assays. 4-EP monooxygenase was measured at 25°C in incubations containing 30 mM phosphate buffer (pH 7.0), a suitable amount of monooxygenase (10 μg of CM arsenate in 100 μM flavin adenine dinucleotide (FAD), 3 μl of FAD reductase (FpdB), 180 mM L-cysteine (Fluka), 2.5 mM NADH, and 400 to 600 μM substrate. Reactions were started by adding NADH. Samples of 25 ml were taken with intervals of 5 to 25 min and were quenched by addition of high-performance liquid chromatography (HPLC) eluent (see below). The samples were centrifuged, and the supernatants were analyzed by HPLC. One unit of enzyme activity corresponds to 1 μmol of 4-EP converted per min.

Flavin reductase activity was determined by monitoring the oxidation of NADH at 340 nm (εmax = 6.22 mM−1 cm−1). Reaction mixtures contained 50 mM phosphate buffer (pH 7.5), 300 μM NADH, and 100 μM FAD or flavin mononucleotide (FMN). The reaction was initiated by addition of enzyme, and initial rates were used for calculating kinetic parameters.

Analytical methods. Isocratic HPLC of 20-μl samples was carried out using a LiChrospher 100 RP8 reversed-phase column (250 mm by 4.6 mm; particle size, 5 μm) in connection with Jasco PU-980 pumps, a Jasco MD-910 diode array detector, and a Jasco UV-2075 detector. The mobile phase (1 ml min−1) was 70:30 (vol/vol) acetic acid-methanol containing 0.02 M ammonium acetate, pH 4.5.

For gas chromatographic (GC) analysis, samples (300 μl) were extracted with an equal volume of ethyl acetate containing mesitylene as the internal standard, followed by analysis on a Hewlett-Packard 6890 GC equipped with a flame ionization detector and an HP-inert Capillary column (Agilent, Santa Clara, CA) (27). Helium (1 ml min−1) was the carrier gas, and the temperature schedule was 5 min at 50°C, followed by an increase of 15°C per min to 250°C.

Fluoride, chloride, bromide, and nitrite were measured in 50-μl samples using a Dionex DX 120 ion chromatograph (Dionex, Sunnyvale, CA) equipped with an Alltech A-2 anion column (100 by 4.6 mm; particle size, 7 μm) and an Alltech guard column (50 by 4 mm). The eluent was a mixture of NaHCO3 and Na2CO3 in deionized water with a flow rate of 1.2 ml min−1.

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been deposited in the DNA Data Bank of Japan under accession no. AB350680 and AB350681.

RESULTS

Cloning of 4-EP monooxygenase genes. In order to obtain the 4-EP degradation genes from strain IF1, we first used a PCR approach. Using degenerate primers, designed on the basis of the alignment of six published gene sequences for two-component aromatic monooxygenases (15, 22, 27, 30, 40), a 731-bp product was obtained. Its nucleotide sequence revealed similarity with 4-nitrophenol (39) and 4-chlorophenol monooxygenase genes (35). Using a large-construct isolation kit, two plasmids were detected in strain IF1. Southern hybrid-

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ization with a probe derived from the PCR-amplified monoxygenase gene fragment indicated that the corresponding genes are not located on these plasmids (data not shown) but on genomic DNA (see Fig. S1 in the supplemental material). Hybridization analysis of genomic DNA restricted with ApaI, BamHI, EcoRI, HindIII, or PstI gave two positive signals in each case, whereas four bands were obtained when the DNA was restricted with SacII. The fact that the probe contained only one Sall restriction site indicates that strain IF1 has two highly similar or identical copies of the 4-FP monoxygenase gene, with different flanking regions.

Based on the hybridization results, two DNA libraries were constructed and screened for the presence of the 4-FP monoxygenase by PCR, which yielded a positive clone from each library. The sequence of the insert of an ApaI clone consisted of 5,145 bp (cluster A). For a BamHI clone, an insert of 9,373 bp was found (cluster B). BLAST sequence similarity searches with the deduced amino acid sequences of the ORFs identified a number of homologs, allowing annotation (Fig. 1; see also Tables S1 and S2 in the supplemental material). Both clusters have a large segment that is similar to a p-nitrophenol catabolism gene cluster (GenBank accession no. EF052871) of Arthrobacter sp. strain JS443 (39). The putative genes involved in 4-FP degradation were designated fpd.

Each cluster contained a putative monoxygenase gene, and these were designated fpdA1 and fpdA2 for cluster A and B, respectively. They are 93% identical at the DNA sequence level and 98.9% identical at the deduced amino acid sequence level. The closest homologs of which the function is established are the hydroxylase proteins of the two-component 4-nitrophenol dioxygenases, e.g., the dioxygenase and the hydroxylase component of similar monooxygenases from Arthrobacter sp. strain JS443 (39), and R. opacus SAO101 (27) (47% identity). Analysis of FpdB with the Pfam database showed the presence in the N terminus of a flavin reductase-like domain (Pfam01613), characteristic for proteins that provide FADH2 to the hydroxylase component. The C-terminal segment of FpdB aligns weakly with the N-terminal segment of GntR-type transcriptional regulators (Pfam00392), indicating the presence of a C-terminal regulator domain.

Some other genes presumably involved in haloaromatic metabolism were detected (Fig. 1). In cluster A, the fpdD and fpdE ORFs encode proteins with sequence similarity to maleylactate reductases that are involved in the degradation of p-nitrophenol (27, 39) and to α/β-hydrolase fold family enzymes, respectively. The translated sequences from ORFs5 and ORF6 in cluster A showed the highest similarity to proteins involved in conjugational plasmid transfer (Pfam02534.12). In cluster B, ORF fpdC encodes a putative protein with high similarity to hydroxyquinol dioxygenases, e.g., the dioxygenase involved in 4-chlorophenol degradation (35), and ORF fpdX encodes a putative periplasmic binding protein. An ORF designated fpdR is present in front of the fpdA2 ORF and may encode a transcriptional regulator, based on the presence of a nucleotide-binding domain and a helix-turn-helix (HTH) motif. It is similar to putative regulator genes encoded in p-nitrophenol and 4-chlorophenol degradation gene clusters (39, 35). Finally, ORF fpdT2, with unknown function, occurs at a similar position in the p-nitrophenol gene cluster (39).

Expression and induction of fpdA1 and fpdA2. The expression of the fpdA genes was tested with strain IF1 cultures exposed to aromatic compounds and with controls. The results

![FIG. 1. Organization of the ORFs in the fpd gene regions of Arthrobacter sp. strain IF1. Open arrows indicate the size and direction of each ORF. ORFs that are expected to be involved in 4-FP metabolism are labeled with fpd. Heavy solid lines delineate regions of high similarity between cluster A and cluster B; heavy dashed lines delineate regions of high similarity between the clusters indicated and the 4-nitrophenol gene region (39); heavy dotted line delineates regions of lower similarity between cluster B and the 4-nitrophenol gene region.](image-url)
of Northern hybridization revealed that fpdA1 and fpdA2 were not expressed in cells incubated with glycerol, glucose, or succinate (see Fig. S2 in the supplemental material), whereas RNA corresponding to the fpdA1 and fpdA2 genes was clearly observed in cells exposed to 4-EP. With 4-nitrophenol as an inducer, the signal was weaker than with 4-EP-grown cells, but induction was still visible for fpdA2.

RT-PCR was used to further analyze the expression of the monooxygenase genes (Fig. 2). When the 3′-specific primer for fpdA1 was used with the 5′-specific primer for fpdA2, or when the 3′-specific primer for fpdA2 was used with the 5′-specific primer for fpdA1, no amplification was obtained, indicating that the primers were specific for their target DNAs. RT-PCR analysis showed no expression of the fpd genes when glucose was used as the carbon source, but with 4-EP, transcription of both fpdA1 and fpdA2 was observed (Fig. 2). The sizes of the amplified fragments (1,189 and 599 bp) were in agreement with the primer positions.

The results of Northern blot analysis and RT-PCR indicate that the expression of fpdA1 and fpdA2 is stimulated by the presence of 4-EP and that fpdA2 is more strongly expressed than fpdA1.

Properties of 4-EP monooxygenase (FpdA2) and flavin reductase (FpdB). To confirm the activities of the proteins encoded by the putative monooxygenase genes, fpdA2 and fpdB were expressed in E. coli BL21(DE3), yielding proteins with molecular masses of approximately 62 and 30 kDa. FpdA2 was purified by a protocol that involved two chromatographic steps (Table 1). Solutions of purified FpdA2 were colorless and showed no absorption in the region of 320 to 500 nm, suggesting that FpdA2 does not contain a flavin cofactor. The reductase component (FpdB) was also purified by column chromatography, after which only one band was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. S3 in the supplemental material). Gel filtration chromatography indicated that FpdB behaved as an octamer. The FpdB protein used NADH to reduce either FAD or FMN (Table 2) but did not use NADPH or riboflavin as a substrate.

In the presence of reductase, the activity of the purified hydroxylase was 160 nmol/min · mg of protein. Thus, the enzyme would have to be present in strain IF1 at a level of at least 5 to 10% of the total cellular protein in order to allow for the observed growth rate (µ = 0.1 h⁻¹), assuming a yield of about 50 mg of cells per mmol of fluorophenol consumed (13).

Conversion of phenols by FpdA2 and FpdB. The purified FpdA2 in combination with FpdB transformed various substituted phenols, with the release of the respective anions (Table 3). No transformation was observed in the absence of NADH or FpdB. The highest transformation rate was observed with 4-bromophenol, whereas 4-chlorophenol, 4-EP, and 4-nitrophenol were converted at lower rates. The disappearance of the para-substituted phenols was accompanied by the release of more than 90% of the substituent as halide or nitrite anions and the formation of hydroquinone (Table 3). Slow conversion of hydroquinone was also observed, both in incubations where it was formed from a 4-substituted phenol and in incubations where it was added from the start, explaining why the amount of hydroquinone detected was smaller than the amount of phenol converted. Some formation of trihydroxybenzene was seen by HPLC when hydroquinone was added (Table 3), but the instability of trihydroxybenzene, leading to brown products, made it impossible to establish mass balances, and rates were too low to conclude that FpdA2 is responsible for hydroquinone metabolism. 4-Nitrocatechol was not formed from 4-nitrophenol or degraded by purified FpdA2 and FpdB.

| TABLE 1. Purification of FpdA2 from E. coli BL21(DE3)(pET17b) |
|-----------------|-----------------|-----------------|-----------------|
| Step            | Total vol (ml)  | Total protein (mg) | Total enzyme activity (U) | Sp act (U mg⁻¹) | Yield (%) | Purification (fold) |
| Cell extract    | 38              | 1,350            | 9.5              | 0.007          | 100       | 1                |
| DEAE Sepharose  | 15              | 250              | 7.5              | 0.030          | 79        | 4.3              |
| Hydroxypatite   | 7               | 38               | 6.0              | 0.160          | 63        | 23               |

a Reaction mixtures contained 400 µM 4-EP, a suitable amount of FpdA2, and the components given in Materials and Methods.

<table>
<thead>
<tr>
<th>TABLE 2. Kinetic parameters of FpdB</th>
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</thead>
<tbody>
<tr>
<td>Fixed substrate⁵</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>FAD NADH</td>
</tr>
<tr>
<td>FMN NADH</td>
</tr>
<tr>
<td>NADH FMN</td>
</tr>
<tr>
<td>NADH FAD</td>
</tr>
</tbody>
</table>

a Values refer to the varied substrate and are means for triplicate experiments with standard deviations.

⁵ FAD and FMN concentrations were fixed at 100 µM, and the NADH concentration was fixed at 300 µM.

<table>
<thead>
<tr>
<th>TABLE 3. Conversion of 4-substituted phenols by purified FpdA2 and FpdB</th>
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</thead>
<tbody>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>4-EP</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
</tr>
<tr>
<td>4-Bromophenol</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
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<tr>
<td>Hydroquinone</td>
</tr>
</tbody>
</table>

a Reaction mixtures contained 600 µM substrate, 0.15 mg ml⁻¹ of 4-EP monooxygenase, and the components described in Materials and Methods. Aromatics were analyzed by HPLC or GC at different times, and anions were analyzed by ion chromatography.

b After 20 min of incubation.

c Hydroxyhydroquinone detected by GC.
The 4-FP degradation genes from *Arthrobacter* sp. strain IF1 were detected on two different but related gene clusters. Cluster A harbors *fpdA1DE*, which comprise a FADH$_2$-dependent monooxygenase, a putative maleylacetate reductase, and a hydrodrolase gene, while in cluster B monooxygenase, a putative maleylacetate reductase, and a hydroxynic compound is induced during growth on 4-FP, as indicated by Northern hybridization and RT-PCR, in agreement with a role in 4-FP metabolism. When 4-nitrophenol was the inducer, only the *fpdA2* gene was well expressed, indicating that FpdA1 is not primarily involved in 4-nitrophenol metabolism in strain IF1. A recent proteomics study showed that the homologous 4-chlorophenol hydroxylase is induced in 4-chlorophenol- and 4-nitrophenol-grown cells of *Arthrobacter chlorophenolicus* (45a).

Comparison of the regions flanking the *fpdA* genes led to the identification of a segment of about 290 nucleotides that is present upstream of the monooxygenase genes in both clusters and that probably was copied together with the monooxygenase gene in a duplication event. Since the sequence present in cluster A is more complete (larger) than the sequence found in cluster B, it is likely that cluster A is more complete (larger) than the sequence found in cluster B. Since the sequence present in cluster B was copied from (a predecessor of) cluster A during the assembly of the clusters. Thus, cluster A may have served as the source of DNA segments for a new cluster that is involved in 4-FP transformation. The FpdA2 and FpdB proteins, purified from *E. coli* transformants, were capable of catalyzing hydroxylation reactions with 4-substituted phenols and released the corresponding anions. The kinetic properties of FpdB (Table 2) are comparable to those of other flavin reductases. The $k_{cat}$ is lower than that of the reductase of a trichlorophenol monooxygenase (TcpX) (4), but the kinetic parameters are similar to those of the reductase of a chlorophenol 4-monooxygenase system (TsTID) (17).

On the basis of genetic and biochemical information, we propose a pathway for 4-FP degradation by *Arthrobacter* sp. strain IF1 that starts with monooxygenation at the para position, with the release of fluoride (Fig. 3). We and others observed the formation of hydroquinone during the transformation of 4-FP (13), 4-chlorophenol (3), or 4-nitrophenol (25, 39). However, if one NADH molecule is consumed per monooxygenase catalytic cycle with anion release, the aromatic product should be benzoquinone. Reduction of benzoquinone to hydroquinone is possible, and a distinct enzyme for this conversion has been proposed for the trichlorophenol (4), pentachlorophenol (10), and 4-nitrophenol (51) degradation pathways. Strain IF1 may have such a protein, but apparently it is not required for the formation of hydroquinone in vitro, since we also detected this product with purified enzymes. Chemical reduction of a quinone by reduced nicotinamide cofactors has been suggested for 2,6-dichlorobenzoquinone (10, 17) and 6-chlorohydroxybenzoquinone (4, 49), and we observed NADH oxidation with benzoquinone (data not shown). Hydroquinone was slowly converted both by whole cells (13) and by mixtures of FpdA2 and FpdB, with some formation of hydroxyhydroquinone, but at this stage we are not certain
about the physiological significance. For the trichlorophenol (4, 49) and 4-nitrophenol (39) pathways, a hydrolysis reaction catalyzed by the initial monooxygenase was proposed, but we consider it impossible to convert benzoquinone to hydroxybenzoquinone in this way, because the stoichiometry does not fit.

The product undergoing ring fission in strain IF1 most likely is hydroxyquinol (trihydroxybenzene), formed either by hydroxylol (4, 49) and 4-nitrophenol (39) pathways, a hydrolysis reaction about the physiological significance. For the trichlorophenol 7772 FERREIRA ET AL. APPL. ENVIRON. MICROBIOL. pathways for bacterial degradation of PCP. Biodegradation via a hydroquinone pathway by tetrachlorobenzoquinone reductase (PcpD). J. Bacteriol. 1998. Genes for 2,4,5-trichlorophenoxyacetic acid metabolism in Burkholderia cepacia AC1100: characterization of the tcf and isd genes and locations of the tcf operons on multiple replicons. Appl. Environ. Microbiol. 64:2086–2093.


2,4,6-trichlorophenol via chlorohydroxyquinol in *Ralstonia eutropha* JMP134 and JMP222. J. Basic Microbiol. **40:**243–249.


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